Transcriptional inducers of acetylcholinesterase expression as novel antidotes for protection against chemical warfare agents

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ABSTRACT

The biological effects of organophosphorous chemical warfare agents (CWAs) are exerted by inhibition of acetylcholinesterase (AChE), which blocks the hydrolysis of acetylcholine leading to hypercholinergy, seizures, *status epilepticus*, respiratory/ cardiovascular failure and death. Current investigations show that bio-scavenger therapy, using purified fetal bovine AChE and the more recently tested human BChE, is a promising treatment for protection against CWA exposure. Impediments such as the complex structure of AChEs, posttranslational modifications, poor yield, and the large amounts of serum required for purification and high-dose regimens for treatment have necessitated a need for alternative bio-scavenger approaches. We investigated the effects of transcriptional inducers to enhance the expression of AChE to achieve sufficient protection against OP poisoning. Trichostatin A (TSA), an inhibitor of histone deacetylase that de-condenses the chromatin and thereby increases the binding of transcription factors and mRNA synthesis, was evaluated for induction of AChE expression in various neuronal cell lines. Dose-response curve show that a concentration of 165 nM TSA is optimal in inducing AChE expression. In Neuro 2A cells, TSA at 165 nM increased the extra-cellular AChE level approximately 3-4-fold and intracellular enzyme 10-fold. Correlating with the AChE induction, TSA pretreatment significantly protected the cells from the cytotoxicity of organophosphate, diisopropyl flurophosphate exposure. These studies indicate that transcriptional inducers, such as TSA, up-regulate AChE, which then can bio-scavenge the organophosphates, and protect the cells from OP induced cytotoxicity, and are potential new ways to treat CWA exposure.

INTRODUCTION

The biological effects of organophosphorous (OPs) nerve agents are exerted by reversible or irreversible inhibition of acetylcholinesterase (AChE), which results in decreased catalysed hydrolysis and excessive accumulation of extracellular acetylcholine (ACh). The ensuing hyperactivation of ACh receptors results in various toxic effects including hypersecretions, convulsions, respiratory distress, coma, and ultimately death. Early, aggressive medical therapy is the key to prevention of the morbidity and mortality associated with nerve agent poisoning. Current emergency treatment of acute OP poisoning consists of combined administration of an AChE re-activator (an oxime), a muscarinic ACh receptor antagonist (e.g. atropine), and an anticonvulsant (e.g. diazepam) (1;2). Recent investigations show that prophylactic enzyme therapy using purified fetal bovine AChE or human plasma butyrylcholinestrase BuChE appears to be a more promising therapy against OP exposure (3;4). Concerns associated with the complex structure of the cholinesterases, posttranslational modifications, genetic variations, intense labor, large amounts of serum needed for purification, low yield of purified enzyme and the high-dose regimens required for therapy has necessitated a search for alternative approaches to nerve agent bioscavanger therapy. One new approach is to induce the expression of endogenous cholinesterases using transcriptional inducers. Transcriptional upregulation of cholinesterases is also useful to produce large quantities of enzyme for purification in addition to complementing the studies on the regulation of cholinesterase expression,

Accumulating evidences suggest that the cellular expression of AChE is regulated both transcriptionally and posttranscriptionally. The promoter of human AChE is activated by cAMP-dependent pathway and augmented by cAMP (5;6). Forskolin, a strong inducer of intracellular cAMP, also has been shown to induce AChE expression. Several binding sites of vitamin D3

and 17β-estradiol have been reported in human AChE promoter (7) and both these factors enhance transcription of the AChE gene. Constitutively activated $G\alpha_s$ and activation transcription factor-1 showed 10- and 4-fold increase in AChE activity. The RNA-binding protein HuD upregulates AChE posttranslationally by binding to the 3′ untranslated region. Calcitonin gene-related peptide induced AChE synthesis in chick myotubes (8). Agonists of P2Y(1) receptors and peroxisome proliferator-activated receptor gamma strongly induce the expression of AChE in neuroblastoma cells (9;10). Antennapedia homeobox peptide causes strong activation of AChE (11). Neuronal-glial interactions mediated by interleukin-1 also significantly increase the expression of AChE (12). All these pieces of literature indicate that the expression of AChE can be significantly increased by transcriptional inducers.

We investigated the effects of transcriptional enhancer, trichostatin A (TSA), to upregulate the endogenous cholinesterases to detoxify OPs. TSA induces gene expression by enhancing the binding of transcription factors through inhibition of histone deacetylase that leads to increased histone acetylation and decondensing of the chromatin (13;14). We show that TSA significantly enhances the expression of both intracellular and extracellular AChE in neurobalstoma cell lines. A similar effect of TSA was also found on BChE expression. Furthermore, the increase level of chlonesterases in TSA treated cells significantly protected the cells from organophaosphate diisopropylfluorophosphate (DFP) toxicity suggesting that transcriptional inducers of cholinesterases are potentially useful for the treatment of nerve agent exposure.

MATERIALS AND METHODS

***Neuronal cell lines and cell culture*:** Mouse neuroblastoma cell lines Neuro 2A was obtained from American Type Culture Collection (Manassas, VA). Neuro 2A cell lines were cultured in

minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (FBS). The cells were cultured at 37° C in a incubator with humidified air and 5% CO_2.

Depletion of cholinesterases from fetal bovine serum*:* To minimize the interference during induction and enzyme assay, serum cholinesterases were depleted using affinity chromatography using procainamide immobilized gel that inhibits serine proteases. Briefly, 10 ml procainamide gel was packed in a glass column and washed with 0.1 M phosphate buffer, pH 8.0. 100 ml of fetal bovine serum was loaded on the column. The first 20 ml of the flow-through that may be diluted with the phosphate buffer was discarded. The remaining 80 ml of the flow-through was collected and filter sterilized by passing through 0.22-0.45 micron filters. Depletion of the serum AChE was confirmed by enzyme assay of the original serum and depleted serum.

Analysis of the effect of transcriptional inducers on AChE expression: Cells were plated (100,000 cells/well/ml) in 48-well tissue-culture plates using AChE depleted serum medium. Trichostatin A (TSA) was diluted in AChE depleted medium and various doses of the compounds as indicated were added to the cells and incubated for different time periods. The medium was changed every three days and fresh TSA was added.

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) microassay: AChE was determined by Ellman's method that was adapted for 96-well plates (15;16). For the AChE assay, an increase in absorbance was monitored at 412 nm for 10 min in a reaction mixture containing 20-50 µl enzyme, 10 µl of 30 mM acetylthiocholine iodide (ATC), 10 µl of 10 mM dithionitrobenzene, and 50 mM sodium phosphate buffer (pH 8) in a final volume of 300 µl. For the BChE assay, butyrylthiocholine substrate was used under similar conditions.

Culture supernatant (20 µl) was used as the source of extracellular enzyme. For the estimation of intracellular AChE, the medium was removed and the cells were washed with phosphate buffered saline (PBS) and lysed using ice-cold phosphate buffer containing 1% Nonidet P-40 or with a lysis buffere purchansed from Sigma. The homogenate was centrifuged for 10 minutes at 3,000 x g at 4°C and the resulting supernatant was used for the intracellular enzyme assay.

Protein determination: Total protein content of the cell lysate was determined by Coomassie brilliant blue protein-binding assay using bovine serum albumin as standard (Bradford, 1976) (Pierce Chemical Co, IL).

DFP treatment and MTT cytotoxicity assay: DFP was diluted in AChE depleted medium, added to the cells and incubated for 3 days. Neuronal cell survival was quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which yields a blue formazan product in living cells but not in dead cells or their lytic debris (17). The resulting colored end product was solubilized in 70% isopropanol containing 2% HCl and the absorbance was measured at 620 nm using the microplate reader.

Cell morphology analysis by microscopy: Changes on the cell morphology was analyzed by microscopy using Nikon microscope (20X).

RESULTS

Depletion of serum AChE by procainimide gel affinity chromatography: To reduce the interference and determine proper expression of AChE following induction with transcriptional enhancers, the serum AChE was depleted using procainamide gel affinity chromatography. A column chromatography was carried out to minimize the time of interaction between serum and procainamide to prevent non-specific binding and elimination of other important serum factors (Fig. 1A). High levels of AChE present in the serum could skew and mar the results or exhibit

feed back inhibition of enzyme expression. Enzyme microassay shows that the AChE was completely depleted from the FBS by this protocol (Fig. 1B). Although the BChE level is very low in fetal bovine serum, a similar result was found with BChE after procainamide affinity chromatography (Data not shown). To further show that procainamide does not leech out from the column and inhibit the expressed AChE, we have incubated equal volume of untreated FBS and procainamide treated FBS and assayed the AChE activity. As shown in Fig. 1C, the AChE value of the mixture was half the value of the untreated serum suggesting that procainamide does not leech out from the column nor inhibit the enzyme during assay.

Trichostatin A upregulates AChE expression in Neuro 2A cell line: Neuro 2A cells were (100 x106 cell/well in 48 well plates) incubated with 0.33, 3.3, 33, 333, and 3333 nM trichostatin A for 7 days. Microscopy results showed that cell morphology of Neuro 2A was altered in TSA treated cells (Fig 2A). Production of distinct filamentous projections was observed at 0.33 nM, 3.3 nM, 33 nM and 333 nM. The neurofilaments started forming networks in 33 and 333 TSA treated cells. TSA was cytotoxic to Neuro 2A cells above 333 nM.

The extracellular AChE expression in the culture supernatant and the intracellular AChE was measured as described in 'Materials and Methods'. The data show that extracellular AChE level was gradually increased in TSA treated cells. The optimum induction of approximately 2-fold was observed at 333 nM TSA treatment (Fig. 2B). Higher dose was cytotoxic to Neuro 2A cells and consequently the level of induction of AChE was low. The intracellular AChE level was also gradually increased in Neuro 2A cells treated with TSA (Fig. 2C). Consistent with the extracellular AChE level, highest induction of AChE, approximately 50-fold compared to untreated controls, was observed at 333 nM TSA treated cells. Total cellular protein level was marginally decreased in TSA treated cells (Fig 2D). The decrease in the total protein may be the

result of reduced cell proliferation during cell differentiation and neurite outgrowth as well as cytotoxicity of TSA at higher doses. Further testing of the effect of TSA showed that 167 nm TSA was the most effective dose for inducing AChE expression (data not shown). We have also found that TSA induces AChE expression in another mouse neuroblastoma cell line, P19 and a human neuroblastoma cell line SH-SY5Y (data not shown).

Effect of trichostatin A on butyrylcholinesterase expression: Although butyrylcholinesterase expression is very low in neuro 2A cells, TSA treatment increased the extracellular expression of BChE levels. Consistent with AChE expression, optimum induction of BChE expression was observed at 333 nM (Fig 3A). Similarly, the intracellular BChE was also significantly increased in TSA treated cells. The induction of intracellular BChE expression was highest at 333 nM TSA treatments (Fig 3B).

Effect of organophosphate diisopropylfluorophosphate in Neuro 2A cells: To study the protection of TSA treatment on the cytotoxic effect of organophosphate exposure, we analyzed the effect of a surrogate organophophate marker, diisopropylfluorophosphate on Neuro 2A cell. The cells ($100X10^5$ cells/well in 48 well plates) were incubated with 0.24, 0.48, 0.95, 1.9, 3.8 and 7.6 M DFP for 3 days. A strong cytotoxicic activity of DFP was visible by rounding, floating of dead cells, and reduction in the number of cells. The cell morphology showed that significant cytotoxicity was evident at the lowest dose of 0.24 M DFP (Fig 4A). MTT cytotoxicity assay results indicated that LD 50 of DFP on neuro 2A cells was 0.19 M (Fig 4B). Extracellular AChE determination showed a decrease in the level of the AChE level with an increase in the dose of DFP (Fig 4C).

Trichostatin A Treatment protects the cells form the cytotoxicity of organophosphate diisopropylfluorophosphate: Neuro 2A cells (100×10^6 cell/well in 48 well plates) were

incubated with 16.5, 33, 86, 167, 263, 333 nM trichostatin A for 7 days. DFP (0.7mM was added and the cells were incubated for 3 days. Microscopy results showed that many of the cells treated with 87 and 167 nM TSA followed by DFP survived and retained cellular morphology (Fig 5A)). Cell survival was maximum at 167 nM TSA. The protection of TSA against DFP cytotoxicity was measured by MTT cytotoxicity assay. Consistent with the cellular morphology, the cytotoxicity data show that 87 and 167 nM TSA treatment significantly protected the Neuro 2A cells against DFP exposure (Fig 5B). However, extracellular AChE is completely inhibited in DFP treated cells (Fig 5C). These data suggest that induction of cellular AChE by TSA protects against the cytotoxicity of organophosphate exposure.

DISCUSSION

The major finding in this study is that potent nerve agent bioscavangers like acetylcholinesterase or butyrylcholinestare can be induced in the cells using transcriptional enhancers. The induced AChE or BChE was able to protect the cells against the cytotoxicity of a surrogate organophosphate marker diisopropylfluorophosphate. These results indicate that transcriptional inducers of AChE or BChE could be used as novel antidotes for the treatment of chemical warfare agents which are potent inhibitors of cholinesterases. Although drugs based on transcriptional regulation are still being investigated and in the process of development for many disorders that are caused by differential gene expression, our study supports a treatment option for organophosphates and opens new avenues for the treatment of chemical warfare agent exposure. In fact TSA is beeign investigated as potential drug for the therapy of lupus (18;19).

The approach of using transcriptional inducers for nerve agent exposure has several advantages. Currently, purified fetal bovine serum AChE or human plasma BChE is being used for the treatment of OP exposure. Causing bodily induction of these two enzymes will minimize

the production of antibodies formed due to the complex structure and posttranslational modification of cholinesterases. Also the cost of drug, labor, and treatment will be much lower by using transcriptional inducers compared to purified enzyme treatments that need lots of human plasma while producing a low-yield. Another advantage of using transcriptional inducers is that the enzyme level can be maintained high as long as the inducer is present in the body and will not need invasive methods of administration.

To accurately measure the induced level of AChE/BChE expression, we have depleted the fetal bovine serum of AChE/BChE using procainamide gel affinity chromatography. Initial experiments with normal fetal bovine serum showed the presence of very high AChE levels that interfere with the estimation of induced AChE because of the high Vmax value. Procainmide gel completely removed the serum cholinesterases. However, protein estimation showed that few other proteins or factors are removed non-specifically by procainamide gel. Accordingly, cell growth was found be normal in procainamide treated serum compared to untreated serum.

In addition to the Neuro 2A cell line, we have done a few experiments with another mouse neuroblastoma P19 cell line and a human neuroblastoma cell line SH-SY5Y. More experiments are needed to prove the potential of AChE expression in mammalian cells by transcriptional inducers. Similarly more experiments with longer incubation with TSA and protection by different concentrations of DFP are required to study the exact dose of TSA that can protect the highest lethal-dose of DFP or other organophosphate surrogate markers. Although we do not expect TSA to induce any other reducing agents containing-SH groups, further study is needed. Alternatively, AChE estimation of the total AChE mRNA by RT-PCR will provide strong evidence of transcriptional induction. In summary, we show that expression of nerve agent bioscavengers like AChE and BChE can be induced by transcriptional inducers

such as trichostatin A. The induced enzyme can bioscavenge the organophosphates, protect the cells from OP induced cytotoxicity, and are potential new ways to treat CWA exposure.

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LEGENDS TO FIGURES

FIGURE 1. Depletion of serum cholinesterases using procainamide gel affinity chromatoigraphy. **A.** Flow-chart depicting the procainamide gel affinity chromatography of fetal bovine serum. **B.** AChE enzyme assay show that procainamide gel removes most of the AChE activity present in the serum. Results are expressed as mean ± SEM (n = 4). **C.** Mixing the procainamide depleted serum with untreated serum does not decrease the enzyme more than half suggesting that procainmide does not come out of the column that may potentially inhibit the expressed AChE.

FIGURE 2. Effect of trichostatin A on Neuro 2 A cells. **A.** Photomicrographs (20X) of Neuro 2A cells treated without or with various doses of Trichostatin A for 7 days. **B.** Extracellular AChE expression determined by microassay of 20 μl culture supernatant. **C.** Cells were lysed with 1% nonidet-P40 and the intracellular AChE expression was determined by using 20 μl cell lysate. D. Total cellular protein in Neruo 2A cells treated without and with various doses of TSA. All the assays were performed in triplicates or quadruplicates and multiple times. The variations between triplicates were less than 10% of the mean.

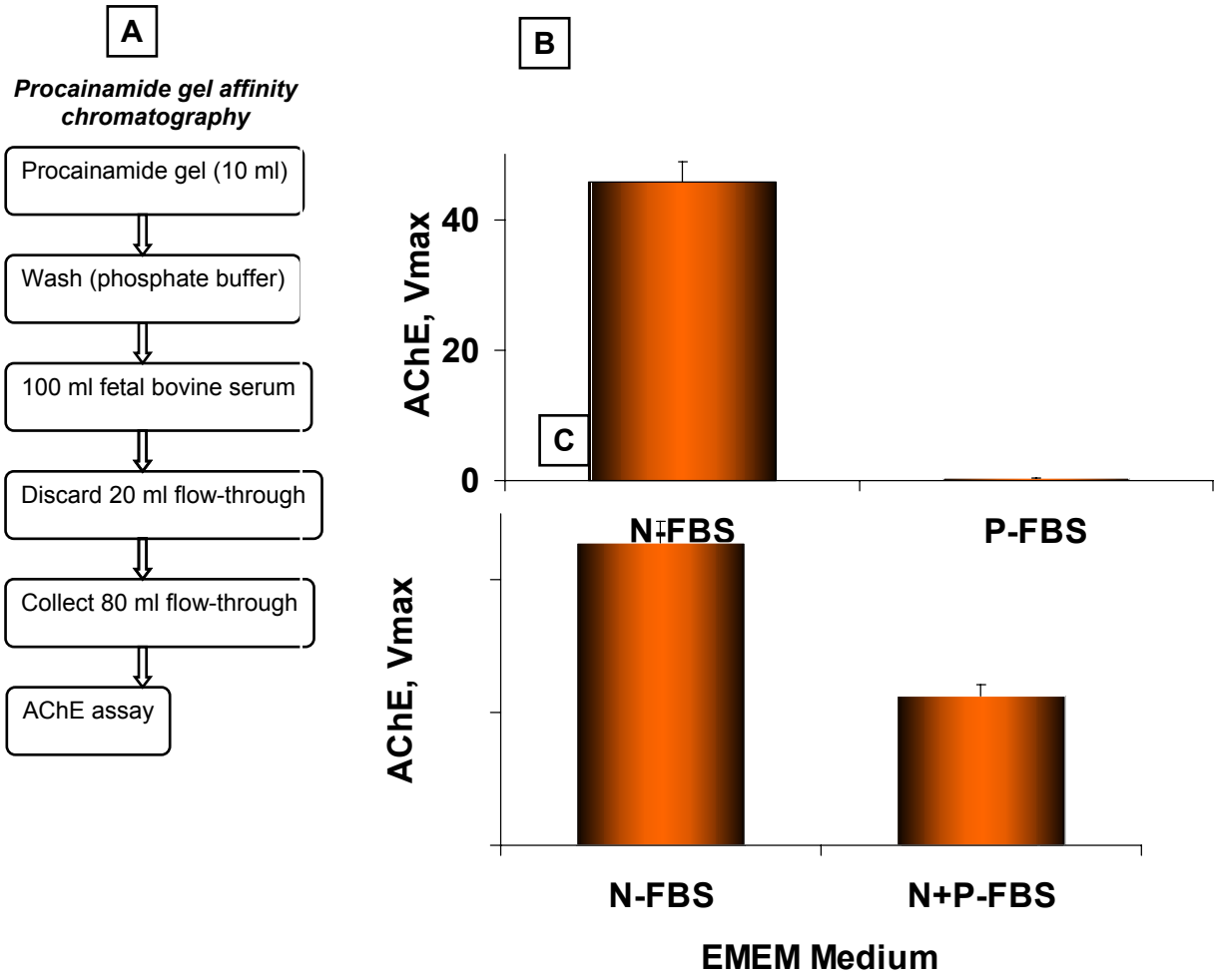
FIGURE 3. Effect of trichostatin A on butyrylcholinesterase expression in Neuro 2 A cells. Neuro 2A cells treated without or with various doses of Trichostatin A for 7 days as described in Figure 2. **A.** Extracellular BChE expression was determined by microassay of 20 μl culture supernatant. **B.** Cells were lysed with 1% nonidet-P40 and the intracellular BChE expression was determined by using 50 μl cell lysate. All the assays were performed in triplicates or

quadruplicates and multiple times. The variations between triplicates were less than 10% of the mean.

FIGURE 4. Effect of diisopropylfluorophosphate on Neuro 2 A cells. A. Photomicrographs (20X) of Neuro 2A cells treated without or with various doses of DFP for 3 days. **B.** The cytotoxicity of DFP was measured by MTT assay as described in 'Materials and Methods' and represented as percent of control. **C.** Extracellular AChE expression was determined by microassay of 20 μl culture supernatant. All the enzyme assays were performed in triplicates. The variations between triplicates were less than 10% of the mean.

FIGURE 5. TSA protects neuro 2A cells from the cytotoxicity of diisopropylfluorophosphate. A. Photomicrographs (20X) of Neuro 2A cells treated without or with indicated doses of TSA and then incubated with 0.78 mM DFP for 3 days. **B.** The protective effect of TSA was measured in DFP treated cells by MTT cytotoxicity assay as described in 'Materials and Methods'. The protection was expressed as percentage of control cells not treated with DFP and TSA. **C.** Extracellular AChE expression was determined by microassay of 20 μl culture supernatant. All the assays were performed in triplicates. The variations between triplicates were less than 10% of the mean.

Figure 1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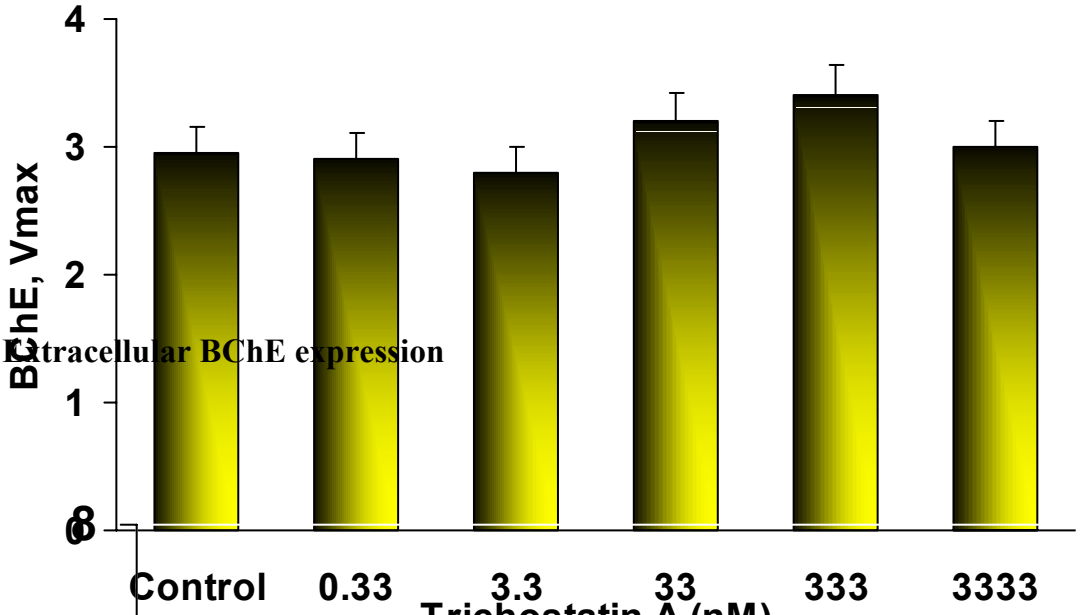
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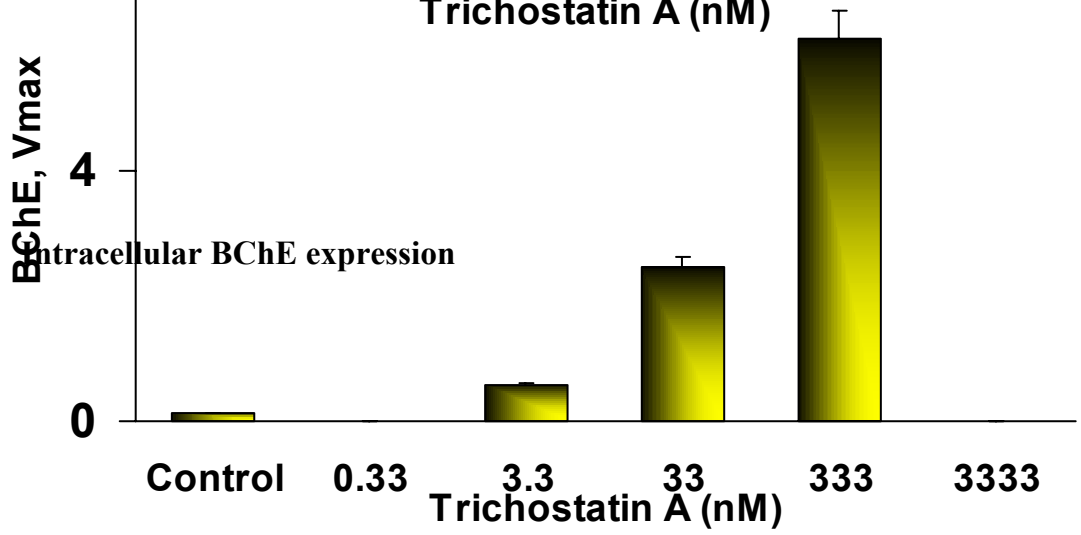
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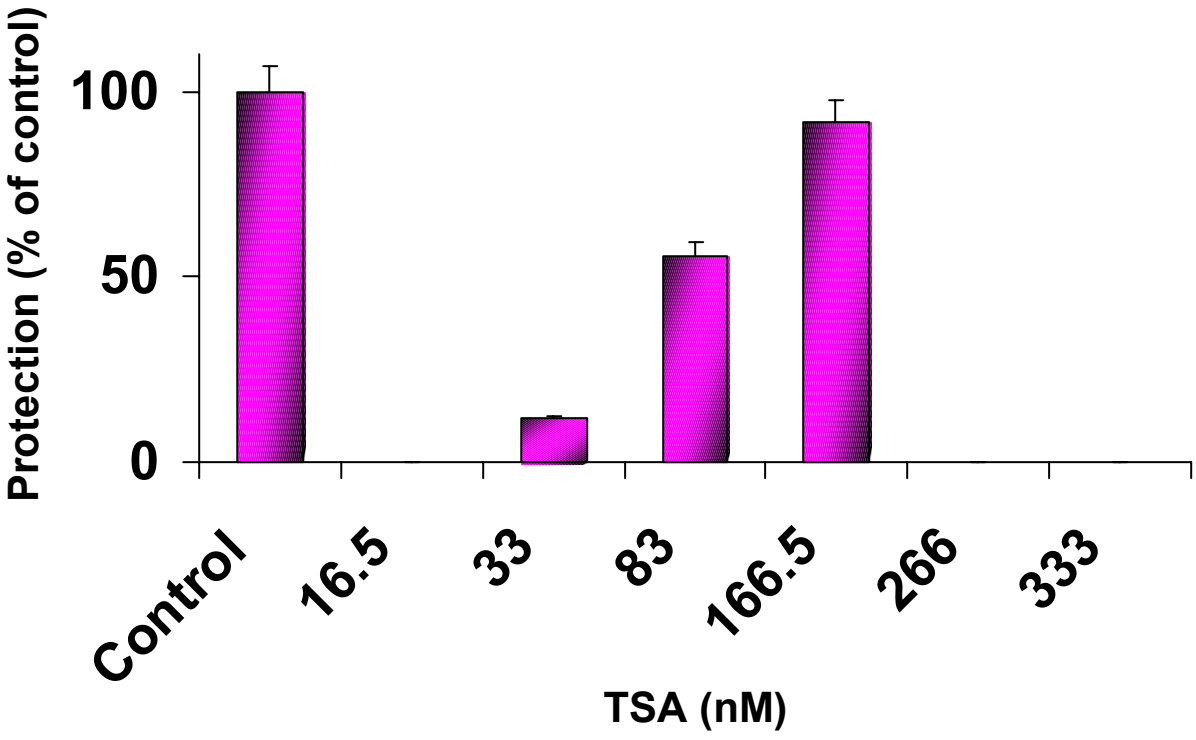
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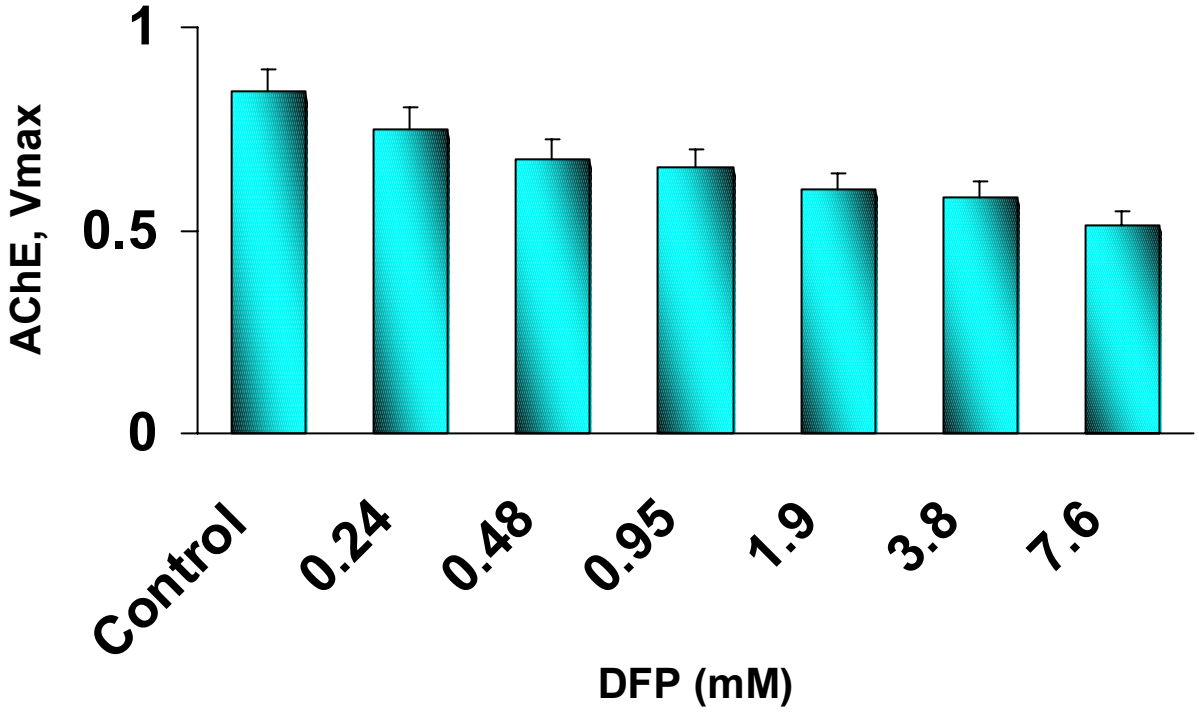
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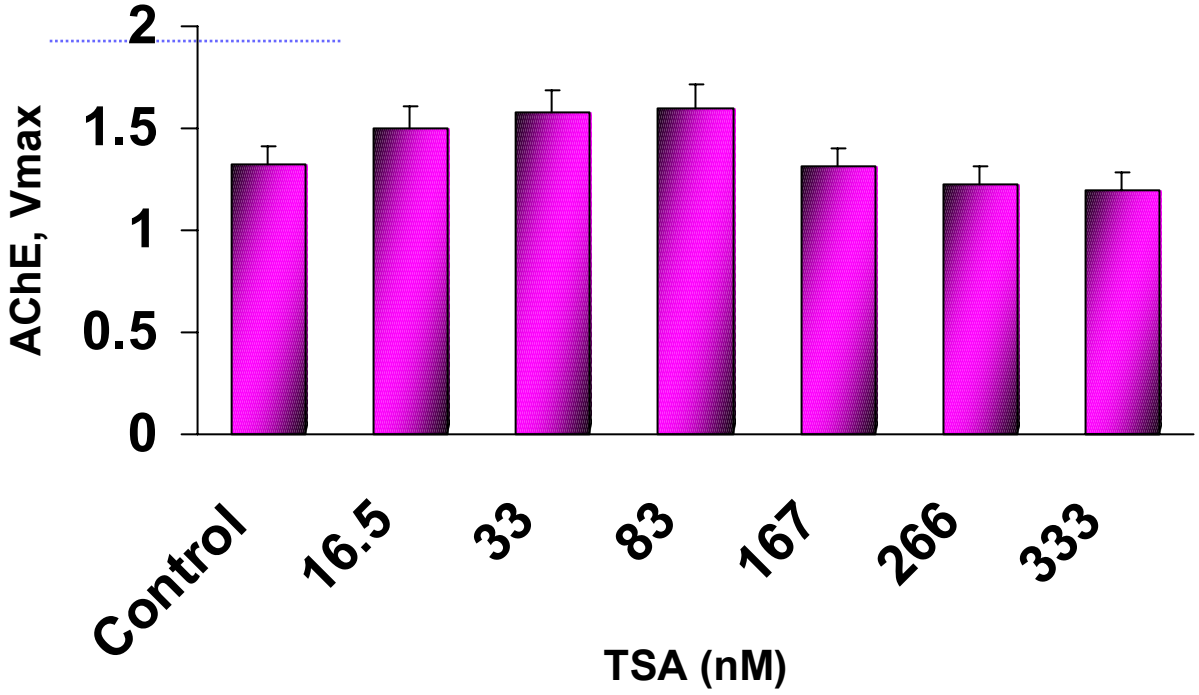
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